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IMMUNOLOGICAL TOLERANCE TO COXSACKIE B3 VIRUS

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The phenomenon of immunological tolerance is attracting the attention of many investigators and is being intensively studied. However, this study is based mainly on the use of noninfectious antigens. Infectious antigens and, particularly, viruses have been little investigated. Only the phenomenon of tolerance which was described in lymphocytic choriomeningitis by Traub [3] is well-known. It consists in the fact that mice born of infected females are not capable of producing antibodies which neutralize the virus of lymphocytic choriomeningitis. This condition is connected with the prolonged retention of virus in the organism of tolerant animals. This observation was used by Burnet [1] in a discussion of questions of the clonal-selection theory of antibody production which he and Erne introduced.

Experiments conducted on white mice with one representative of the enteroviruses – Coxsackie B3 virus, are described in the present article. The results of these studies showed the possibility of reproducing the phenomenon of viral immunological tolerance by infecting females in different periods of pregnancy with this virus. There are indications in the work of Flamm [2] and Surjus [4] of an interuterine infection caused by Coxsackie viruses in mice.

MATERIAL AND METHODS

Females in different periods of pregnancy were used for the development of immunological tolerance. In order to determine tolerance the progeny of these mice were immunized at the age of 15 days and older. Both the pregnant females and the mice born from them were injected with Coxsackie B3 virus (Strain F-5 which we isolated in Fryazino in 1958). The virus titer was determined from the number of cytopathogenic doses and the titrated suspension was stored at -20°. The virus was grown on transplanted pig embryo kidney cells (PES). The pregnant females were injected intravenously with 1 ml of culture liquid containing various amounts of CPD_{50} for PES cells. For immunization of tolerant and

control animals 1 ml (10^7 CPD_{50}) of the virus was injected intraperitoneally. Blood was taken on the 7th day for antibody determination. When there were two immunizations the interval between the virus injections was 7 days. The serum from each group of identical mice was mixed, heated at 60° for 20 min and stored at -20° until the beginning of the experiment. The presence of antibodies in the serum was determined by a neutralization reaction on PES cells and additionally on transplanted human amnion cells. Before testing the mixture of serum and virus was kept at room temperature for 1 hour.

RESULTS

After injection of pregnant females with Coxsackie B3 virus the newborn mice became sick on the 2nd to 4th day and died from a specific infectious process. The specificity of the process, as a rule, was confirmed histologically and pathological changes typical of Coxsackie B3 virus were found in the brain, brown fat, liver, pancreas and skeletal muscles (individual myositic foci). However, this infectious process did not always develop and depended on the amount of virus injected into the females. With a decrease in the doses of injected virus some of the newborn mice survived and with minimal infectious doses, the progeny was born without any signs of pathology.

TABLE 1

Results of Intravenous Injection of Coxsackie B3 Virus Into Pregnant[1] Females

1 Число самок, получивших вирус	2 Количество введенного вируса (в $ЦПД_{50}$)	3 Число родившихся мышей	4 Число павших мышей	5 Число выживших мышей в потомстве	6 День гибели животных
10	100 000	64	64	0	5—6-й 8
18	10 000	154	37	117[2]	7—9-й 8
10	1000	87	14	73	5—6-й 8
5	600	30	0	30	—
8	100	84	0	84	—
8	10	69	0	69	—
Контроль (10)	—	80	0	80	—

1) The period of pregnancy was 17-19 days for infected mice.
2) In 61 out of the 117 mice the defects were congenital (growth retardation).

1) Number of females which received virus; 2) amount of virus injected (in CPD_{50}); 3) number of mice born; 4) number of mice which died; 5) number of surviving mice in progeny; 6) day of animals' death; 7) control; 8) th.

TABLE 2

Dependence of Tolerance on Dose of Virus Injected Into Pregnant[1] Females

Число самок, получивших вирус (1)	Доза введенного вируса (в $ЦПД_{50}$) (2)	Число иммунизированных мышей (3)	Средний титр антител после иммунизации (4)
20	10 000	100	0
20	1000	100	0
4	600	30	0
4	100	36	0
3	10	18	1:64
Контроль 5	—	100	1:256

1) Period of pregnancy of infected females 19 days.

1) Number of females which received virus; 2) dose of virus injected (in CPD_{50}); 3) number of immunized mice; 4) average antibody titer after immunization; 5) control.

TABLE 3

Dependence of Viral Tolerance on Period of Pregnancy

1 Число зараженных самок	2 Срок беременности (в днях)	3 Доза введенного вируса (в $ЦПД_{50}$)	4 Число иммунизированных мышей	5 Средний титр антител на 7-й день после вакцинации
4	2—4	1000	28	0
8	9—14	1000	45	0
20	16—20	1000	100	0
Контроль 6			100	1:256

1) Number of infected females; 2) period of pregnancy (in days); 3) dose of virus injected (in CPD_{50}); 4) number of immunized mice; 5) average antibody titer on 7th day after vaccination; 6) control.

TABLE 4

Duration of Immunotolerance

Число зараженных самок A	B День беременности	Доза введенного вируса (в $ЦПД_{50}$) C	Титр антител у мышей (потомства), иммунизированных D в различные сроки после рождения (в днях)						
			15	20	30	40	50	60	90
8	19-й E	10^3	0	0	0	0	0	0	1:16
8	Контроль F		1:16	1:64	1:128	1:64	1:64	1:128	1:64

A) Number of infected females; B) day of pregnancy; C) dose of virus injected (in CPD_{50}); D) antibody titer of mice (progeny) immunized in various periods after birth (in days); E) 19th; F) control.

TABLE 5

Dependence of Viral Tolerance on Number of Vaccinations

A Номер зараженной самки	1-я вакцинация B		2-я вакцинация C	
	D число мышей	титр антител на 7-й день	D число мышей	титр антител на 7-й день E
1	5	0	5	0
2	4	0	4	0
3	3	0	4	0
4	3	0	4	0
F Контроль	5	1:32	5	1:128

Note: The period of pregnancy of the infected mice was 19 days, the dose virus injected was 10^3 CPD_{50}.

A) Number of infected females; B) 1st vaccination; C) 2nd vaccination; D) number of mice; E) antibody titer on 7th day; F) control.

TABLE 6

Dependence of Viral Tolerance on Antigen Quality

1 Антиген, введенный самкам (срок беременности 17—19 дней)	2 Число самок	3 Доза введенного вируса (в $ЦПД_{50}$)	4 Минимальная иммунизирующая доза (в $ЦПД_{50}$)	5 Число иммунизированных мышей (потомство)	6 Титр антител у иммунизированных мышей (потомство)
Живой вирус Коксаки B3 7	10	10^4	10^3	100	0
Инактивированный нагреванием 8	12	0	10^4	60	1:64
Формалинизированный 9	7	0	10^6	44	1:128

1) Antigen injected into females (period of pregnancy 17-19 days); 2) number of females; 3) dose of virus injected (in CPD_{50}); 4) minimal immunizing dose (in CPD_{50}); 5) number of immunized mice (progeny); 6) antibody titer of immunized mice (progeny); 7) living Coxsackie B3 virus; 8) inactivated by heating; 9) formalinized.

TABLE 7

Immunotolerance to Different Viruses. Specificity of Phenomenon of Immunological Tolerance

1 Вирус, введенный беременным самкам	2 Вирус, использованный для иммунизации мышей (потомство)				
	3 энтеровирусы			4 грипп	
	Коксаки B3 5	Коксаки B5 6	полиомиелит типа I 7	A1	A
Энтеровирусы: 3					
Коксаки B3 5	0/128	64/64	64/64	80/160	40/40
Коксаки B5 6	64/64	0/64	—	—	—
полиомиелит типа I 7	64/64	64/64	0/64	—	—
Вирус гриппа: 8					
A1	—	—	—	0/40	160/160
A	—	—	—	40/40	0/160

Note: Numerator – antibody titer of tolerant mice; denominator – antibody titer of control animals.

1) Virus injected into pregnant females; 2) virus used for immunization of mice (progeny); 3) enteroviruses; 4) influenza; 5) Coxsackie B3; 6) Coxsackie B5; 7) type I poliomyelitis; 8) influenza virus.

Composite data characterizing the dependence of the infection of newborn mice on the doses of virus injected are presented in Table 1. The fact that average doses of virus of 10^3-10^4 CPD_{50} produced, as a rule, regularly recurring results deserves attention; a considerable number of the newborn mice survived but bore traces of an intrauterine infection which was manifested mainly in a sharp lag in the weight and growth of such mice in comparison with the controls. When minimal doses of virus were injected into pregnant females the majority of the mice were born externally healthy but in this case acquired a state of immunological tolerance. And, finally, doses of virus not exceeding 10^3 CPD_{50} did not have any effect on the newborn mice.

All the newborn mice remained under observation for 30 days, then were immunized with Coxsackie B3 virus. Animals of the same age and weight were used for a control. As a rule, all the mice which were born of the infected females were tolerant to injected antigen and did not produce antibodies.

The dependence of the tolerance on the dose of virus injected into the pregnant females is shown in Table 2. It appears that a condition of tolerance develops in mice born from females which received both average and minimal doses of virus. Virus doses not exceeding 10 CPD_{50} proved to be the least effective in this respect. In immunized mice produced by such females an increase in antibodies after immunization was found, but the titer was 4 times less than in the controls.

We did not succeed in determining whether virus is retained in the organism of tolerant mice. The virus was isolated in a culture of PES cells and suckling mice. For this purpose, the blood and organs of pregnant and lactating females as well as the blood and organs of embryos and suckling mice, respectively, were used in experiments conducted in parallel. As a rule, we succeeded in establishing the presence of virus in high titers in the blood and organs of pregnant and lactating females. Virus was not found in material taken at different times from embryos and suckling mice.

In further experiments, we studied some conditions of the development of viral immunological tolerance, the length of retention and the specificity of this phenomenon.

As seen from Table 3, the period of the females' pregnancy at the time of the injection of virus does not have an effect on the birth of tolerant mice. Virus injected into females in the 1st, 2nd and 3rd week of pregnancy caused the same condition of immunological tolerance in the progeny. It should be mentioned that if virus is injected into females before mating, this does not have any effect on the progeny.

The experiments showed that the condition of tolerance is retained by the mice for a long time. The results of an antibody determination in tolerant mice in the course of the 3 months of their life are presented in Table 4. As is seen, antibodies are found only on the 90th day, but in a considerably lower titer

than in the control animals.

Having in mind the rule of immunological reactivity according to which the repeated injection of antigen always causes more intensive antibody production, we conducted experiments with repeated immunization of tolerant mice. Data on the dependence of viral tolerance on the number of immunizations are presented in Table 5. An increase in antibodies was not found after a repeat injection of antigen on the 7th day after immunization, and the reimmunization remained without results.

We further determined the degree to which the quality of the antigen injected into pregnant females affects reproduction of the phenomenon of tolerance and whether inactivated Coxsackie B3 virus produces a similar effect. The results of these experiments are presented in Table 6, from which it is seen that Coxsackie B3 virus inactivated both by heating at 60° for 30 min and by action of formalin (1:4000 for 72 hours at 37°) not only lost its infectiousness but also the capacity to cause a state of tolerance in the progeny. It should be noted that antigens which have lost their infectious activity retained the capacity to cause antibody production in vaccination of the control mice.

In the last series, we determined the specificity of the phenomenon of tolerance. For this, mice born of females which received Coxsackie B3 virus during pregnancy were immunized with Coxsackie B5, type I poliomyelitis, influenza A1 and influenza A (Strain PR8) viruses. The results of this experiment are presented in Table 7.

Mice tolerant to homologous antigen reacted to the same degree as the control animals to immunization with influenza, poliomyelitis and Coxsackie B5 viruses. The results of these investigations make it possible to believe that viral immunological tolerance is a highly specific phenomenon and even enteroviruses so close in physical and chemical properties as type I poliovirus and Coxsackie B5 proved in this case to be heterologous antigens.

The assumptions which we made concerning the possibility of developing immunological tolerance with other viruses deserve attention. As seen from Table 7, it was obtained in mice born of females immunized during pregnancy with influenza A1 and PR-8 virus as well as with poliomyelitis type I and Coxsackie B5 viruses.

DISCUSSION

An intrauterine infection develops in mice infected during pregnancy with Coxsackie B3 virus. The newborn mice either become ill and die from a typical infectious process or have congenital deformities. This second outcome of an intrauterine infection is interesting in that it can serve as a model for the study of some questions of antenatal and neonatal pathology.

A third outcome of an intrauterine infection with Coxsackie B3 virus, in our opinion, is the most interesting: the mice born in this case are immunologically tolerant to homologous antigen.

Here it is appropriate to compare the data which we obtained with the clonal-selection theory of antibody production. It is necessary to assume that the genetic information which determines the specificity of cells' immunological competence is established in the embryo's cells in the first stage of pregnancy. It is determined by intrauterine contact with the virus which injures a specific clone of cells and therefore antigen injected into tolerant mice does not cause the production of specific antibodies. This condition evidently is not connected with survival of the virus in the organism – with the carriage of virus which sharply differentiates it from the immunological tolerance described in lymphocytic choriomeningitis. By the end of the 3rd month the condition of tolerance caused by Coxsackie B3 virus is gradually lost, which indicates the existence of compensatory processes or, in other words, reconstruction of the clone of immunologically competent cells responsible for the production of antibodies against Coxsackie B3 virus.

The high degree of specificity of viral immunological tolerance draws attention. Even a virus so close in biological and physicochemical properties as Coxsackie B5 virus is a heterologous antigen for tolerant mice and causes antibody production.

Although we did not succeed in isolating Coxsackie B3 virus from the organism of tolerant mice, nevertheless it should be recognized that a state of immunological tolerance can be caused only by a virus capable of multiplication. This is proved by the fact that Coxsackie B3 virus inactivated by heating or by the action of formalin when injected into pregnant females was devoid of the capacity to evoke a state of immunological tolerance. We do not consider the experiments which we conducted sufficient to resolve the question of the absence of virus from tolerant mice. It has not been excluded that virus exists in the organism in one or another noninfectious form which cannot be demonstrated by the methods which we used.

CONCLUSIONS

1. When pregnant mice are injected with specific doses of Coxsackie B3 virus their progeny develops a state of immunological tolerance. This state is retained for up to 3 months and gradually is lost.

2. The state of immunological tolerance to the virus is highly specific. Tolerant mice respond to the injection of other viruses with as much antibody production as the control mice.

3. Other supplementary data connected with intrauterine virus infection are presented in the paper and the obtained results are discussed.

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IMMUNOLOGICAL TOLERANCE TO COCKSACKIE B-3 VIRUS

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SUMMARY

When mice were inoculated with Coxsackie B3 virus at different periods of pregnancy, intrauterine transmission of infection occurred. As a result of inoculation of large virus doses, newborn mice developed the typical infectious process terminating in death of the animals. Some surviving mice showed congenital anomalies.

When females were inoculated with reduced virus doses, newborn mice developed immunological tolerance The study determined maximum and minimum amounts of virus, required for reproduction of this process.

Mice born to infected mothers retained the acquired tolerance for 8-9 weeks. Immunization performed after this time resulted in antibody response but to a considerably lower titer than in control animals.

Attempts at virus isolation from suckling mice born to infected mothers were unsuccessful which did not permit to attribute the state of immunological tolerance to prolonged survival of the virus in the body. However, inoculation of pregnant females with heat-inactivated virus had no results. The lack of effect in this case indicated the importance of an infectious process in formation of immunological tolerance.

Inoculation of tolerant mice with Coxsackie B3 virus revealed no difference in susceptibility between experimental and control animals.

Inoculation of tolerant mice with a suspension of the thymus or spleen from normal mice one hour before immunization stimulates specific antibody production, indicating partial elimination of areactivity to the antigen.